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ARTICLE

Spectrum of mutations and genotype–phenotype analysis in Currarino syndrome

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The triad of a presacral tumour, sacral agenesis and anorectal malformation constitutes the Currarino syndrome which is caused by dorsal-ventral patterning defects during embryonic development. The syndrome occurs in the majority of patients as an autosomal dominant trait associated with mutations in the homeobox gene *HLXB9* which encodes the nuclear protein HB9. However, genotype–phenotype analyses have been performed only in a few families and there are no reports about the specific impact of *HLXB9* mutations on HB9 function. We performed a mutational analysis in 72 individuals from nine families with Currarino syndrome. We identified a total of five *HLXB9* mutations, four novel and one known mutation, in four out of four families and one out of five sporadic cases. Highly variable phenotypes and a low penetrance with half of all carriers being clinically asymptomatic were found in three families, whereas affected members of one family showed almost identical phenotypes. However, an obvious genotype–phenotype correlation was not found. While *HLXB9* mutations were diagnosed in 23 patients, no mutation or microdeletion was detected in four sporadic patients with Currarino syndrome. The distribution pattern of here and previously reported *HLXB9* mutations indicates mutational predilection sites within exon 1 and the homeobox. Furthermore, sequence homology to *Drosophila* homeobox genes suggest that some of these mutations located within the homeobox may alter the DNA-binding specificity of HB9 while those in sequences homologous to a recently identified NLS motif of the human homeobox gene PDX-1 may impair nuclear translocation of the mutated protein. *European Journal of Human Genetics* (2001) **9,** 599–605.

Keywords: Currarino syndrome; *HLXB9* mutations; mutational predilection sites; DNA-binding specificity; NLS motif; genotype–phenotype correlation

Introduction

The triad of a presacral tumour or an anterior meningocele, a sacral agenesis and an anorectal malformation was first described as a unique congenital complex in 1981.[1] Several observations suggest that Currarino syndrome is caused by malformation of the caudal notocord which leads to aberrant secondary neurulation and an incomplete separation of the endodermal and ectodermal layer in the developing embryo.[2] The syndrome occurs in the majority of patients as an autosomal dominant trait and is associated with mutations in the homeobox gene *HLXB9*.[3] This gene encodes the nuclear protein HB9, that includes a highly conserved homeodomain which shares a 68% homology to the *D. melanogaster* homeobox gene proboscipedia. Precise activation, in space and time, of homeobox genes is essential for normal morphogenesis and has been shown to participate in the regulation of human embryonic development.[4] In addition, high phylogenetic conservation of regulatory mechanisms acting over vertebrate homeobox complexes suggest that minor modifications of these genes or time difference in gene activation might have contributed to morphological variations during evolution.[5] Although famil-

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ial cases of Currarino syndrome have been shown to be caused by *HLXB9* mutations,[3] systematic genotype–phenotype analyses have been performed only in a few families. Furthermore, there are no reports about how HLXB9 mutations specifically might impair HB9 function. In this study we report five *HLXB9* mutations, analyse the genotype–phenotype correlation and describe the variability of phenotypes within those families. In addition, we discuss the distribution of these and previously reported *HLXB9* mutations and, based on homology to other homeodomain genes, we suggest functional implications of some mutations located within the *HLXB9* homeobox.

Materials and methods

Subjects

After obtaining informed consent, 72 individuals from nine unrelated families with Currarino syndrome were included in this study. Four families originate from Germany and one from Yugoslavia, Poland, Italy, Russia and Turkey. Four were multiplex and five simplex families.

Diagnosis was based on clinical findings, radiological investigation and mutational analysis. Index patients and relatives were evaluated by history and physical examination, review of medical records and radiological investigation including plain X-rays, ultrasound imaging, rectal radiography, magnetic resonance imaging (MRI) or computed tomography (CT) of the sacral spine and pelvic region.

Mutation analysis

DNA was extracted from blood samples with standard procedures. For sequencing the coding region, the putative promoter and intron 2 of the *HLXB9* gene, we used previously designed PCR primers.[3] PCR reactions were performed in a total volume of 15 μl, consisting of 1 μl DNA, 6 μl H_2O, 10 pmol of each primer, 10 mM dNTP, 0.5 U polymerase, 1 × polymerase buffer and 2 M betaine, using the Perkin & Elmer GeneAmp PCR System 9600 (Foster City, CA, USA). After an initial denaturation step at 96°C for 2 min, we performed 30 cycles of amplification at 96°C for 10 s, at the optimised annealing temperature (70°C for the first PCR product and 57°C for the second PCR product) for 15 s and at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were purified using the QIAquick purification kit (Qiagen, Hilden, Germany) and sequenced by using the BigDye terminator kit (Perkin & Elmer, USA) and the ABI PRISM™ 310 Genetic Analyzer.

DNA samples of the patients' relatives were analysed for *HLXB9* mutations either by electrophoresis on 2% agarose gels at 70 V for 30 min or on SSCP gels at 280 V for 14–17 h. SSCP gels consisted of 12% polyacrylamide and 1 × TBE buffer, were stained with SYBR gold (Molecular Probes, Eugene, Oregon, USA) for 10 min and subsequently screened with a FluorImmager SI, Vistra™ Fluorescence (Molecular Dynamics, Sunnyvale, CA, USA).

Haplotype analysis

To analyse whether one family of our study was related to a previously described American or a French family[6] which carried the same mutation (130-131insC), we performed haplotype analysis using polymorphic markers cen-D7S550, CGR13, D7S559, CGR16 and D7S2423-tel. Microsatellites were amplified in 15 μl PCR mixtures containing 30 ng DNA, 0.33 μM of each primer, one of which was end-labelled with fluorescent dye (FAM), 0.1 mM of each dNTP, and 0.5 U Taq DNA polymerase (Perkin & Elmer, USA) in 50 mM KCl, 10 mM Tris-HCl (pH 8.0), and 1.5 mM $MgCl_2$. Reactions consisted of 27 cycles at 94°C for 15 s, at annealing temperature for 15 s, and at 72°C for 30 s. Sequencing was performed using an automated DNA sequencer (ALF, Pharmacia, Uppsala, Sweden).

Results

Mutation analysis

In five members of family 1 from Germany we found a frameshift insertion of a cytosine into a stretch of six cytosines at positions 125–130 within the first exon (130–131insC) (Figure 1A). This mutation was previously reported in an American and a French family.[3,6] Haplotype analysis revealed different haplotypes in each of the three families, suggesting a recurrent mutation.

In family 2 of german origin we identified a frameshift insertion of guanine into a stretch of six guanines at positions 408–413 (413–414insG) in 10 individuals (Figure 1A). Interestingly, patient II-2 was mentally retarded and born with anal atresia, but did not show a *HLXB9* mutation. Haplotype analysis confirmed that this patient did not inherit the mutated allele from his mother, indicating that anal atresia in this case was not part of Currarino syndrome.

Sequence analysis in family 3 from Yugoslavia revealed an inframe deletion of 6 bp at position 4285 (4285–4290del) in four family members. This mutation results in loss of threonine and glutamine residues at the 3′ end of exon 2. These two residues are highly conserved and belong to the homeodomain encoding sequence (Figure 1B).

In another german pedigree (family 4) we found a frameshift insertion of a cytosine into a stretch of two cytosines in exon 1 at positions 262–263 (263–264insC) in three patients (Figure 1A).

Mutational analysis of one turkish female sporadic patient showed a frameshift insertion of a 13 bp fragment 5′-GCCCGCCGACCGC-3′ at position 294 (294–307ins). This unique mutation is a duplication of the sequence between positions 281 and 293, probably caused by homologous recombination (Figure 1A).

In contrast, extensive sequencing and microsatellite analysis did not reveal any mutation or chromosomal microdeletion of *HLXB9* in the four remaining sporadic patients with Currarino syndrome, one male and three females from Poland, Russia, Germany and Italy. Since the

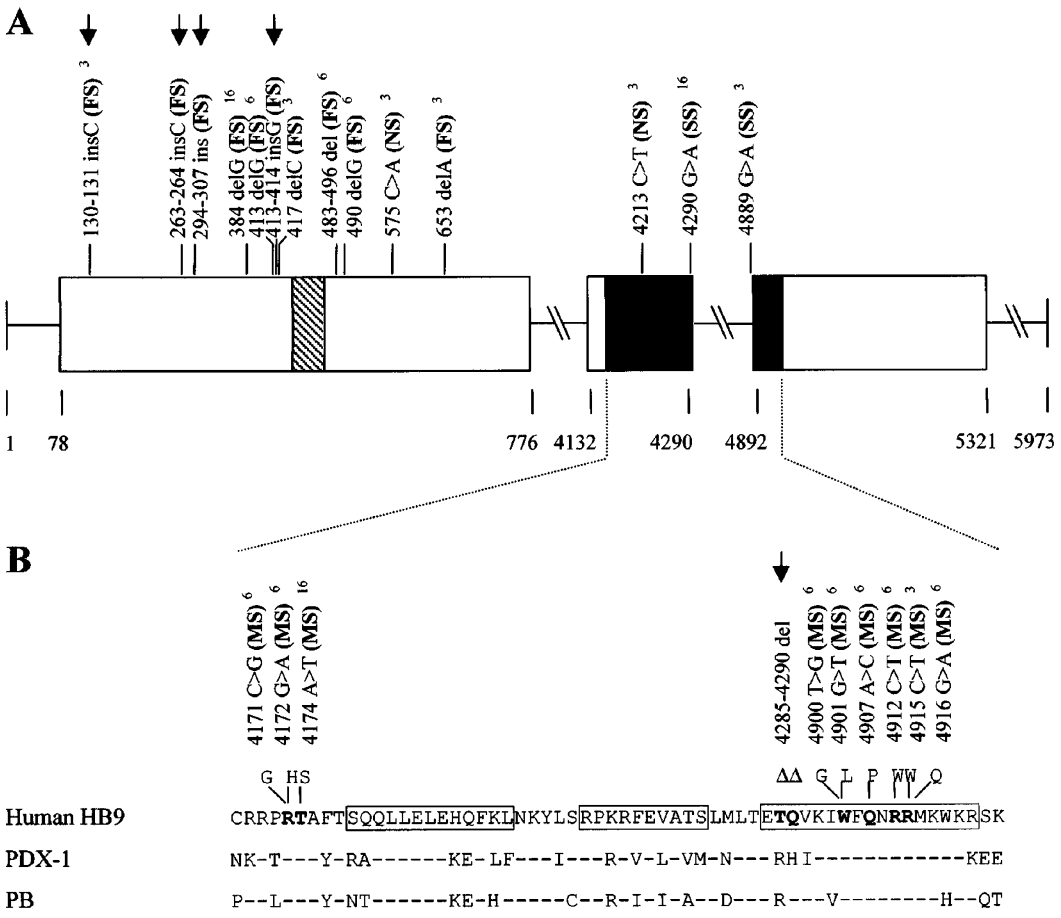

Figure 1 Spectrum of mutations in the *HLXB9* gene. (**A**) Mutational spectrum and genomic organization of the *HLXB9* gene, with coding region depicted by rectangular boxes. Hatched box indicates a poyalanine repeat, and black box the homeodomain. Numbering is based on genomic sequence in bp. All known frameshift mutations (FS), nonsense mutations (NS) and splice site mutations (SS), insertions (ins) and deletions (del) are indicated and references are included for previously reported mutations. Those mutations reported in this study are marked with black arrows. (**B**) Amino acid sequence homology of the human HB9 homeodomain to that from human pancreatic duodenal homeobox-containing transcription factor 1 (PDX-1) and proboscipedia (PB). Dashes denote identity with HB9. Rectangular squares represent the three helices of the HB9 homeodomain. All known inframe mutations are located within the homeodomain and shown for clarity only in (**B**). References are indicated for previously reported mutations. The altered amino acids resulting from missense mutations (MS) are depicted in bold with the mutant residues shown above. The loss of threonine and glutamine residues due to the deletion of 6 bp (4285–4290del) found in this study is indicated (Δ) and marked with a black arrow.

second intron within the homeobox might be of significance for HB9 function, we also sequenced this intron, but did not identify any sequence alteration.

Phenotype analysis

Chronic constipation since early childhood was the predominant symptom and present in 13 of 23 patients with *HLXB9* mutations and in four of four sporadic patients without *HLXB9* mutations. Urinary tract infection was diagnosed in a total of five patients, meningitis in three patients and delivery problems due to the presacral tumour in one patient (Table 1). Although 10 of 23 patients with *HLXB9* mutations did not present any clinical symptom, radiological investigation revealed characteristic phenotypic features in all patients, including those who were asymptomatic (Figure 2). However, the complete triad, consisting of a presacral mass, an anorectal malformation and a sacral agenesis was found only in eight of 23 patients with *HLXB9* mutations, three males and five females. In contrast, none of the four sporadic patients who did not show a *HLXB9* mutation were asymptomatic and the complete Currarino triad was present in three of those patients (Table 1).

A presacral solid tumour and/or an anterior meningocele was found in 15 of 23 patients with a *HLXB9* mutation and in

Table 1 Phenotype and *HLXB9* mutations of patients with Currarino syndrome

		Symptoms			Radiological findings											
						Presacral		Anorectal			Sacral				Urogenital	
Patients	*Mutations*	*Constipation*	*Urinary tract infection*	*Delivery problems*	*meningitis*	*Meningocele*	*Solid tumour*	*Rectal stenosis*	*Anal atresia*	*Rectal fistula*	*Sacral defect*	*Tethered cord*	*Intraspinal lipoma*	*Syringocele*	*Vesicoureteral reflux*	*Doubled vagina/ uterus*
Family 1																
I-1	130–131 insC	−	−	−	−	−	+	−	−	−	V	−	+	−	−	−
II-2	130–131 insC	+	−	−	−	−	−	−	−	−	III	+	−	−	−	−
II-4	130–131 insC	+	−	−	−	−	−	−	−	−	V	−	−	−	−	−
III-1	130–131 insC	−	−	−	−	+	+	−	−	−	IV	+	−	−	−	−
III-2	130–131 insC	+	+	−	−	+	−	−	−	−	III	+	+	−	+	−
Family 2																
I-2	413–414 insG	−	−	−	−											
I-6	413–414 insG	−	−	−	−	−	−	−	−	−	IV	−	−	−	−	−
I-7	413–414 insG	+	−	−	−	+	+	+	−	−	IV	+	−	−	−	−
I-8	413–414 insG	−	−	−	−	+	−	−	−	−	IV	−	+	−	−	−
II-4	413–414 insG	−	−	−	−	−	−	−	−	−	V	−	−	−	−	−
II-5	413–414 insG	+	−	−	−	+	−	+	−	−	IV	−	−	−	−	−
II-10	413–414 insG	−	−	−	−	−	+	−	−	−	V	−	+	−	−	−
III-1	413–414 insG	−	−	−	−	−	+	−	−	−	V	−	−	−	−	−
III-2	413–414 insG	+	−	−	−	−	−	−	−	−	IV	+	+	+	−	−
III-5	413–414 insG	+	−	−	−	+	+	+	−	−	IV	+	+	+	−	−
Family 3																
I-2	4285–4290 del	−	−	−	−						IV					
II-2	4285–4290 del	+	−	−	−	+	−	−	−	−	IV	−	−	−	−	−
II-3	4285–4290 del	−	−	−	−	−	−	−	−	−	IV	−	−	−	−	−
III-1	4285–4290 del	+	−	−	−	+	+	+	−	+	IV	−	+	−	−	−
Family 4																
II-2	263–264 insC	+	+	+	−	+	+	+	−	−	IV	+	+	−	−	+
III-1	263–264 insC	+	−	−	+	+	+	+	−	+	IV	+	−	+	−	−
III-2	263–264 insC	+	+	−	+	+	+	−	+	+	IV	+	−	+	−	−
Sporadic																
1	294–307 ins	+	−	−	−	+	+	+	−	−	IV	+	−	+	−	−
2	No mutation	+	−	−	−	+	+	+	−	+	IV	+	+	−	−	−
3	No mutation	+	−	−	+	−	−	−	−	−	IV	+	+	−	−	−
4	No mutation	+	+	−	−	−	+	−	+	+	IV	+	+	+	+	+
5	No mutation	+	+	−	−	+	+	−	+	−	IV	+	+	+	+	−

Presence (+) or absence (−) of symptoms and findings. Patient I-2 from family 2 and patient I-2 from family 3 refused further radiological investigation. The type of sacral defect was classified as follows: Type III: Subtotal sacral agenesis with S1 present; Type IV: Hemisacrum, Type V: Total or subtotal coccygeal agenesis.

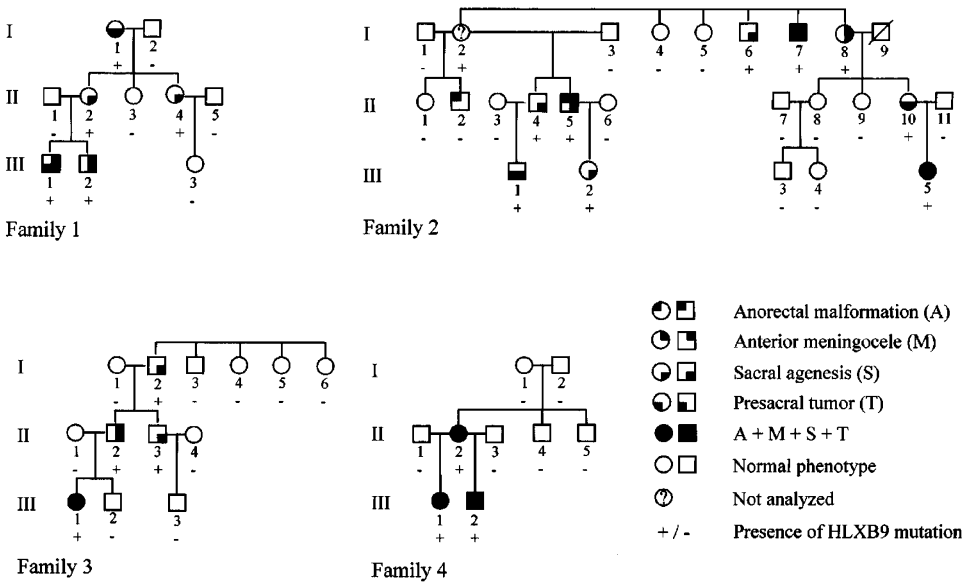

Figure 2 Pedigrees, phenotypes and genotypes of families 1 – 4. The clinical status of family members was evaluated by patients history, X-ray, ultrasound and MRI or CT scans. The presence of phenotypic features as anorectal malformation, anterior meningocele, sacral agenesis and presacral tumour is indicated by shading of different quadrants. Presence or absence of *HLXB9* mutations is indicated by + or −, respectively. Patient I-2 of family 2 refused radiological investigation.

three of four sporadic patients without a *HLXB9* mutation. Anorectal malformations included a rectal stenosis in eight patients, an anal atresia in three patients and a rectal fistula in five patients. The most common radiological finding of Currarino syndrome was a sacral defect, present in all patients with the exception of one patient from family 2 (I-2) who refused radiological investigation. However, there was a remarkable variability of sacral agenesis (Table 1). Coccygeal hypoplasia as minimal variant was found in three patients of family 1 carrying the frameshift mutation (130 – 131insC) and in two patients of family teo carrying the frameshift mutation (413 – 414insG), whereas in patients with other *HLXB9* mutations and in patients without *HLXB9* mutations the extent of sacral agenesis was much more profound. A tethered cord and an intraspinal lipoma was diagnosed in all sporadic patients but only in about half of the familiar cases. Seven of 14 patients with tethered cord additionally showed a syringocele. Vesicoureteral reflux or urogenital malformations were far less common and diagnosed only in three or two patients, respectively (Table 1).

In all familial cases we observed an autosomal dominant transmission (Figure 2). Compared with highly variable phenotypes seen within families 1 – 3, all three affected individuals from family 4 carrying the frameshift mutation (263 – 264insC) showed the complete triad and similar phenotypes (Table 1).

In conclusion, we found a broad spectrum of phenotypes in most families affected by Currarino syndrome. Although some *HLXB9* mutations may be associated with more distinct phenotypes, we did not find obvious phenotype differences between patients from families with different *HLXB9* mutations.

Discussion

A very detailed clinical investigation of all patients failed to identify any obvious genotype – phenotype correlation, similar to previous investigations.[6,8] It seems to be likely that the phenotype in most patients with Currarino syndrome is at least in part determined by a critical nuclear threshold level of HB9. However, the local HB9 concentration during embryonic development is known to be influenced by the negative autoregulatory activity of HB9.[7] Although in Currarino syndrome the expression of HB9 is limited by haploinsufficiency, the degree of transcriptional repression of the non-mutated allele may not be identical for every case with a given mutation, which could explain the enormous intramutational phenotypic variability. Alternatively, modifier genes have been suggested to participate in the formation of different phenotypes.

We identified a HLXB9 mutation in all familial cases. In one sporadic case we found a duplication of a 13 bp fragment (294 – 307ins), most likely due to homologous recombination, while we did not find a *HLXB9* mutation in four other sporadic patients with Currarino syndrome. Only in about 30% of sporadic cases of Currarino syndrome *HLXB9* mutations have been identified and five sporadic patients in whom no mutation was detected have been reported

previously.[6] Since mutations have been detected in most patients with familial Currarino syndrome, somatic mosaicism seems to be the best explanation for the low mutation detection in sporadic cases. However, mutations outside the coding region and partial deletions of the gene may also contribute to this phenomenon. Genetic heterogeneity may be less likely, but cannot be discounted, since there may be another locus at 13q or 20p.[9]

The distribution of these and previously described *HLXB9* mutations suggest mutational predilection sites between positions 125 and 130, a stretch of six cytosine residues, and between positions 408 and 413, a stretch of six guanine residues that might be caused by replication slippage mechanism. The fact that mutation (130–131insC) seen in family 1 was also present in a previously described American and a French family[3,6] and that those families are not related suggest a recurrent mutation. The mutation in family 2 (413–414insG) is located in a region that encodes a polyglycine rich tract. A deletion of guanine at the same position (413delG) was described previously.[6] In addition, a deletion of cytosine was found immediately downstream of this region within a repeat of four cytosines (417 delC) (Figure 1A).[3]

In general, more than half of all *HLXB9* mutations are located within the homeobox. Interestingly, all missense mutations are clustered between position 4171 and 4174 at the N-terminal region and between 4900 and 4916 at the C-terminal region of the homeobox (Figure 1B). The mutation seen in family 3 (4285–4290del) is the first inframe deletion within the HLXB9 homeobox reported so far, since only single amino acid substitutions and one nonsense mutation have been described. The presence of a *lab*-class intron which interrupts the homeobox is an interesting feature of *HLXB9* and is shared with other genes which have the Q'VK protein sequence conserved at this position.[10] The 6 bp deletion is located immediately C-terminal of exon 2 and alters the conserved Q'VK protein sequence but does not change the intron-exon splicing consensus sequence.

All reported *HLXB9* mutations to date directly or indirectly affect the DNA binding homeodomain. While the predominant type of mutation N-terminal of the homeobox is a single base pair frameshift insertion or deletion, all inframe mutations are located within the homeobox and consist in most cases of single base pair replacements. Since alleles with frameshift mutations N-terminal of the homeobox encode proteins that lack the homeodomain, they represent loss of function alleles, leading to HB9 haploinsufficiency. However, inframe mutations within the homeobox might affect HB9 function in a more specific way. The homeodomain contains three helices with the third being the DNA-recognition helix. The majority of *HLXB9* homeobox mutations alter the protein sequence of the third helix which is the most conserved region of the HB9. It shares 14 of 17 residues with several other homeodomain proteins including *D. melanogaster* homeobox gene poboscipedia (pb) which shows the most similar homeodomain to HB9 (Figure 1B). Previous studies of variants with changes at position 50 have suggested a key role for this residue in DNA binding specificity.[11,12] Thus, the *HLXB9* missense mutation 4907 A>C (Q50P), described previously,[6] may alter the DNA binding specificity of HB9. On the other hand, recent structural studies have suggested a more complex picture. Although glutamine is the most common residue at position 50, a variety of side chains occupy this position in the homeodomain family[13] and glutamine is suggested to have only a minor impact on specificity via water mediated contacts.[14] Alternatively, Q50P may also completely abolish DNA binding through disruption of the alpha-helical structure of the recognition helix.

Recently, a novel type of nuclear localisation signal (NLS) was identified within the homeodomain of human pancreatic duodenal homeobox-containing transcription factor 1 (PDX-1).[15] The NLS motif RRMKWKK is almost identical to residues 52-58 within helix 3 of the HB9 homeodomain (RRMKWKR) (Figure 1B). In addition, the residue lysine at position 46 which enhances nuclear transport of PDX-1 is present in both genes. Three mutations have been described 4912 C>T (R52W), 4915 C>T (R53W) and 4916 G>A (R53Q), which alter one of the two arginine residues at positions 52 or 53 within the NLS motif.[6,16] Mutation of both residues in PDX-1 result in complete cytoplasmic retention.[17] Thus, these residues may also play a role for nuclear translocation of HB9 and the above mentioned *HLXB9* missense mutations potentially reduce nuclear translocation of HB9. Alternatively, mutations at either positions may also impair DNA binding ability, as have been shown in the CRX and HESX1 homeodomains.[18,19]

Polyalanine repeats are an increasingly recognized motif at the N-terminal side of both homeodomain proteins and in non-homeodomain transcription factors and might be associated with transcriptional repressor activity.[20] The size of the polyalanine repeat has been reported to be correlated with gain of function in other homeobox genes which influence the disease phenotype.[21,22] However, no mutation has been reported within the region encoding the polyalanine repeat in HB9, so far, and variation in length is not associated with the presence of Currarino syndrome.[16]

In summary, several conclusions can be drawn from this study.

(1) Mutational analysis of nine index patients and relatives with Currarino syndrome revealed five mutations N-terminal or within the homeobox of *HLXB9*, four novel and one known mutation, in four out of four families and in one out of five sporadic cases. In all familial cases we observed an autosomal dominant genotype transmission.
(2) Although 10 of 23 individuals with *HLXB9* mutations were clinically asymptomatic, altered phenotypes were seen in all patients with Currarino syndrome. However, the complete triad was present only in eight cases with *HLXB9* mutations and in three sporadic cases without a

HLXB9 mutation. With the exception of one family, we found a low phenotypic penetrance and a broad spectrum of phenotypes in patients who carried the same mutation. The predominant symptom in affected individuals was chronic constipation since early childhood. However, like other investigators we did not find an obvious genotype–phenotype correlation.

(3) The distribution of here described and previously reported *HLXB9* mutations indicates mutational predilection sites within exon 1 and within the homeobox. Furthermore, sequence homology to *Drosophila* homeobox genes and to the human homeobox gene PDX-1 suggests that some mutations within the *HLXB9* homeobox may alter the DNA binding specificity while other mutations may reduce nuclear translocation of the mutated protein.

(4) In general both, autosomal dominant transmission and low clinical penetrance of Currarino syndrome indicate that the number of patients affected by *HLXB9* mutations has been underestimated, so far. Therefore, the syndrome should already be considered when only a partial phenotype is present. In all patients who present with a history of chronic constipation since early childhood a pelvic X-ray should be performed to test for sacral agenesis, the most common sign of Currarino syndrome. In those patients with any form of sacral agenesis, a mutational analysis should be considered in order to offer adequate genetic counselling.

Electronic-database information

Accession numbers and URLs for the data in this article are as follows:

GeneBank, http:\\www.ncbi.nlm.nih.gov

Online Mendelian Inheritance in Man (OMIM), http:\\www.ncbi.nih.gov/omim MIM 176450.

Acknowledgments

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